RESPONSE OF PEARL MILLET INBREDS AND HYBRIDS TO INOCULATION WITH *Spirillum lipoferum*

BY

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Dedicated to the memory of my grandfather,

Basilio Fratesi; a farmer, a man.

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Inoculation experiments were conducted on six hybrids and 15 inbreds (including the hybrid parents) of pearl millet, *Pennisetum americanum* (L.) K. Shum., using the N_2-fixing bacterium, *Spirillum lipoferum* Beijerinck (Sp 13t), as liquid inoculum. The objective of this study was to investigate reconstitution of N_2-fixation among these inoculated plant genotypes by measuring enhancements of dry weight, percent nitrogen, total nitrogen and acetylene reduction (nitrogenase) activity.

One hybrid, Tift 23DA X Tift 186 ('Gahi 3'), gave significantly ($p = 0.05$) higher dry weight yield in response to field inoculation. Inoculated plots of 'Gahi 3' produced 32% more dry weight and 37% more total plant nitrogen when compared to killed inoculum controls.

No inbred was found to respond as vigorously as 'Gahi 3', but one, Bil 3B, was enhanced 17% in dry weight by inoculation. Acetylene reduction values were low (range 0 - 54 nmole/g dry root x hr) and did not support yield effects. This assay was not used extensively because of the damage it would cause plant agronomic yields. The possibility exists that high levels of acetylene reduction were not measured due to the limited sampling.

A nitrogen balance study was conducted in the greenhouse in large ceramic containers on inoculated 'Gahi 3' plants to repeat the yield differences observed in the field and to monitor inputs of nitrogen into the soil-plant system. Increases of 3.2% in total plant dry weight ($p = 0.28$) and 4.3% in total plant nitrogen ($p = 0.21$) were observed when compared to autoclaved inoculum controls. No increase of nitrogen into the soil-plant system was found due to inoculation. A sampling error of 3.48% was calculated and any associative N_2-fixation achieved could have been within the limits of this sampling error.

It is concluded that differences do exist among pearl millet genotypes to respond to bacterial inoculum with increased agronomic yield, but sensitive assays such as $^{15}N_2$ incorporation must be used to confirm N_2-fixation in these responders. Further screening for associative N_2-fixation achieved through inoculation should place initial emphasis on well replicated experiments designed to show increased nitrogenase activity by extensive sampling with acetylene

reduction or, if possible, $^{15}N_2$ incorporation. Those genotypes which are deemed responders should then be field tested individually for increases in agronomic yield and acetylene reduction.

Roy L. Smith
Chairman

INTRODUCTION

Biological nitrogen fixation (N_2-fixation) by plants in association with prokaryotic organisms is one of the most important natural processes on the earth today. The legume-*Rhizobium* symbiosis is such an association where N_2-fixation is responsible for fulfilling the plant's nitrogen requirement for growth and production. Inoculation with *Rhizobium* bacteria to achieve N_2-fixation is well known and facilitates its agricultural use. Associative plant-microorganism N_2-fixing systems are found in agricultural soils. These groups are believed to fix appreciable quantities of nitrogen worldwide. An associative N_2-fixing bacterium, *Spirillum lipoferum* Beijerinck, was isolated from the roots of various tropical grasses and showed potential for contributing nitrogen to this group of plants. Plant genetic variation to associate with *S. lipoferum* was predicted (21). Any plant variability offers excellent potential to enhance associative N_2-fixation by use of plant breeding.

The purpose of this study was to measure differences among inbreds and hybrids of pearl millet, *Pennisetum americanum* (L.) K. Shum., to reconstitute N_2-fixing associations with *Spirillum lipoferum*, Sp13t, inoculum. Pearl millet is a diploid tropical forage and cereal grass in which selfing and hybridization is easily achieved. Variability within this grass to associate with bacterial inoculum for increased N_2-fixation is an important plant breeding consideration.

REVIEW OF LITERATURE

Associative N_2-Fixation

Crop plants require a great deal of nitrogen for maximum growth and production. Each hectare of land is surrounded by an atmosphere containing over 80,000 MT of nitrogen yet none is directly useful to plants. This atmospheric nitrogen must be reduced to usable plant forms. Reducing nitrogen to synthetic fertilizer forms by the Haber-Bosch process is wasteful of nonrenewable energy sources, but nitrogen produced by the process increased from 4.5×10^6 MT in 1957 to 36×10^6 MT in 1974. Biological N_2-fixation uses renewable solar energy, and though estimated to contribute 80×10^6 MT of nitrogen per year, its contribution remained constant during the same time (30). Efficient use of biological N_2-fixation to supply nitrogen to agricultural crops is needed as nonrenewable energy supplies dwindle and become more expensive.

The legume-*Rhizobium* symbiosis is an efficient N_2-fixing system. But nitrogen contributed to agricultural land by associative N_2-fixing organisms is considerable. Estimates predict quantities of nitrogen fixed by these systems to approach the 10.8×10^6 MT fixed by legumes in the United States. Available energy limits N_2-fixation by associative systems in soils. Microorganisms which produce energy by photosynthesis, or those which derive energy in association with plants are more likely to fix significant amounts of nitrogen for agricultural crops (44).

Nonleguminous plants, especially the cereal grains, constitute the world's major food producing crops. Associative N_2-fixation shows promise for supplying nitrogen to these crops. Nitrogen-fixing systems are now documented in rice (Cryza sativa L.), maize (Zea mays L.), sugar cane (Saccharum officinarum L.), and several tropical pasture grasses (18, 21, 24, 47). Rates of fixation in rice were reported to be equal to some legumes in flooded paddies (24). The bacterium, Azotobacter paspali, was discovered in close association with bahiagrass, Paspalum notatum Flugge (20). Colonies of A. paspali were found to establish in the root mucigel layer and N_2-fixation rates up to 90 kg N/ha/yr were predicted in this associative system (22). A. paspali is found almost exclusively on broad leaved, pubescent bahiagrass cultivars such as 'Batatais' (33).

Efficient N_2-fixation in 'Transvala' digitgrass, Digitaria decumbens Stent., led to the isolation of strains of the bacterium, Spirillum lipoferum, from the roots of this forage grass (21). Spirillum strains were found to fix nitrogen in pure culture by acetylene reduction, Kjeldahl total nitrogen, and $^{15}N_2$ incorporation (21, 39). Cultures showed optimum temperature for their N_2-fixation to range from 32 to 40 C with little fixation below 24 C or above 42 C (17). The bacterium reduced N_2 only under microaerophylic conditions and optimum pO_2 for acetylene reduction was between 0.006 and 0.02 atmospheres (41). Cell free extracts require both Mg^{2+} and Mn^{2+} for good nitrogenase activity (40). Spirillum lipoferum is a common soil organism and guineagrass, Panicum maximum Jacq., is believed to be the most favored forage grass for isolating the organism (23).

Differences for potential N_2-fixation are found among cultivars of Paspalum and Digitaria (21, 42). Screening maize for N_2-fixing ability by the acetylene reduction assay showed variability between S_1 lines of the rust resistant variety UR-1. The best lines exhibited potential fixation of up to 2 kg N/ha/day and N_2-fixing Spirillum strains were isolated from these active S_1 plants (47). This report indicates genetic variation within maize to interact with Spirillum for associative N_2-fixation. Beneficial colonization of grass roots by S. lipoferum as a criteria in plant breeding warrants investigations.

Inoculating legumes with Rhizobium bacteria facilitates its experimental and agricultural use. Reconstitution of grass-Spirillum N_2-fixing associations through inoculation is therefore important. When used as inoculum in the field, S. lipoferum (strain Sp 13t) produced significantly higher yields of dry matter than did uninoculated controls in field grown, lightly fertilized pearl millet and guineagrass. Up to 42 and 39 kg N/ha were calculated by regression analyses to be replaced by Sp 13t inoculum in these two respective grasses (45). Work in Oregon with Spirillum inoculum (strain Sp 81) on maize showed less than 4 g N/ha/day fixed. Yield and nitrogen content were not enhanced, thus providing no evidence of appreciable N_2-fixation (1). Inoculation responses of enhanced dry matter and total plant nitrogen in buffelgrass, Cenchrus ciliaris L., with Sp 13t inoculum were not repeatable in the second year of testing (6).

Use of bacterial inoculants other than Rhizobium are reported by Brown (7) to be unsuccessful in achieving N_2-fixation. Any beneficial plant yield responses were thought to come from other

modes of action such as production of growth regulating substances, suppression of plant pathogens, or mineralization of soil phosphates. Culture supernatant of the N_2-fixing bacterium Azotobacter paspali was found to contain the plant growth regulators IAA, GA, and cytokinin in appreciable amounts (2). Inoculation studies on bahiagrass plants grown in pots revealed no evidence of N_2-fixation and increases in plant yield were postulated to come from the growth regulating substances secreted by the bacteria. This study was conducted under low light intensities. Achievement of N_2-fixation under high light conditions in the field was not discounted (8).

Nitrogenase Enzyme and Assays of N_2-Fixation

Any study on biological N_2-fixation requires a basic understanding of the nitrogenase enzyme and those assays which allow measurement of the process. More intensive reviews are available on the physiology and biochemistry of the process (4, 10, 48).

Nitrogenase catalyzes the reduction of atmospheric nitrogen to ammonia and the reaction is shown in the following equation (48):

$$N_2 + 6e + 12\ ATP + 12\ H_2O \text{ ---- } 2\ NH_3 + 12\ ADP + 12\ Pi + 4H^+.$$

Thermodynamically the energy demanding step in N_2-fixation is the breakdown of the nitrogen-nitrogen triple bond in diatomic molecular nitrogen. The bond energy required to reduce N≡N to N=N is 126 kcal and is considered large for a biological system (32). Nitrogenase is therefore a high energy electron acceptor. In the electron donor system of the nodule bacteriods, NADPH which is generated by

glucose-6-phosphate dehydrogenase and the electron carriers, ferredoxin and flavodoxin, will couple electrons with nitrogenase (49).

The reduction of N_2 with H_2 is exothermic at one atmosphere and a free energy change of -17.5 kcal/N_2 when 3 H are supplied has been calculated for the system. The following reduction reactions show the exothermic nature of N_2 reduction with the exception of N_2 to the diimide. This tends to support the theory that the diimide is not a free intermediate of the system (32).

Reduction Reaction	H (kcal/mol at 25 C)
$N_2 + H_2$ --- N_2H_2	+44
$N_2H_2 + H_2$ --- N_2H_4	-20
$N_2H_4 + H_2$ --- $2NH_3$	-44

Nitrogen reduction to NH_3 is therefore thermodynamically favored and theoretically should need no input of energy or ATP. Energy is shown to be essential to nitrogenase from ATP saturation curves and must be present in any cell free preparation for completion of the reaction (10). Since most nitrogenase reactions are shown as occurring in two electron steps and expressed as electron pairs transferred;

$$2H^+ + 2e \text{ --- } H_2$$

$$6H^+ + N_2 + 6e \text{ --- } 2NH_3$$

this has led to speculation that ATP is utilized at the rate of 4.0 to 4.6 moles per $2e^-$ (mole of H_2 evolved or 1/3 N_2 fixed). This would give some 6.0 to 6.9 ATP molecules for each ammonium (NH_4^+) ion as this ion is usually the form found in biological systems (5). This need for available energy is thought to be the major limiting factor in the development of associative N_2-fixing systems (44).

Nitrogenase reactions utilizing substrate levels of ATP proceed optimally when Mg^{2+} is supplied at a level which assumes the presence of both an ATP:Mg^{2+} complex and free ATP. But there can be any number of divalent cations used in the following order of importance: Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (10).

After N_2 reduction, the ammonia produced is believed to be assimilated by entering organic combination with keto acids produced via the tricarboxylic acid cycle in one of two ways. With glutamate dehydrogenase participating, the initial major product is glutamate, followed by glutamine. This has been confirmed by ^{15}N tracer studies which showed these compounds to in fact be early labeled products (36). Recently, another pathway of ammonia incorporation in free living systems has been postulated via glutamine synthetase (36, 43). Glutamine synthetase is believed to have the primary role of reacting with the amino acid glutamate and NH_3 produced from fixation to form the amino acid glutamine (43).

Inhibitors of nitrogenase include carbon monoxide, hydrogen, oxygen, and ammonia (32). Nitrogen fixation is an anaerobic process with oxygen inhibiting the activity of nitrogenase (32, 36). Natural systems to protect the enzyme from this element have been evolved. The nodulating bacteriods are surrounded by leghemoglobin to give the enzyme protection from O_2. In free living Azotobacter, O_2 is consumed via cytochrome oxidase system bound to its membrane. But O_2 sensitivity would be another limiting factor in development of associative N_2-fixing systems (44).

Three methods of assaying N_2-fixation are used: Kjeldahl total nitrogen, incorporation of ^{15}N, and acetylene reduction. The Kjeldahl method for total nitrogen determination is slow, destroys the plant and fixed nitrogen is not distinguishable from that obtained from other sources (29). Even with these drawbacks, total nitrogen is a good method for confirming the results of other indirect N_2-fixing assays such as acetylene reduction (27). Alone, the method is satisfactory if done with extensive replications and statistical analyses to show total nitrogen gain in a system (11, 19). A nitrogen increase of 1% needs to take place before differences can be detected (11).

The isotope, ^{15}N, provides a more direct assay method and any increase from the gaseous form ($^{15}N_2$) in the plant gives positive evidence of N_2-fixation (11, 29). It is also not subject to the sampling problems of the Kjeldahl total nitrogen procedure. Accumulation of 0.015 atmo % excess of ^{15}N would require only 0.025% increase in total plant nitrogen, thus it would be 100 times more sensitive than the total nitrogen assay (11). But, ^{15}N is expensive and a mass spectrometer is required in the analysis (27).

An important and extensively used assay method is acetylene reduction (3, 19, 27, 29). It is based on the fact that nitrogenase catalyzes the reduction of acetylene to ethylene (C_2H_2 to C_2H_4) as well as the reduction of N_2 to NH_3 (34). In its simplest form the assay is conducted on the desired plant material or microbial culture in a sealed chamber which has a port for exchanging gases. Acetylene is introduced into the chamber in known concentrations with an inert

gas such as argon and incubated for specific periods of time. A sample is taken and gas fractions separated through a gas chromatograph to measure amounts of ethylene reduced (29).

Acetylene reduction is very useful for investigations into the biochemistry and physiology of nitrogenase and N_2-fixation, as well as defining N_2-fixing systems of soil and plants. Although sensitive to the nanomole range, this assay must be supplemented by other nitrogen determining methods as the 3:1 theoretical conversion ratio (ethylene:nitrogen) is not consistent for all systems (31).

Taxonomy, Breeding, and Cytogenetics in Pearl Millet

Pearl millet dry matter yields were significantly enhanced with Spirillum lipoferum (Sp 13t) inoculation (45). This enhanced response and the ease of manipulating this grass genetically led to its selection for use in this investigation. A review of its taxonomic, breeding, and cytogenetic characteristics is now presented.

Pearl millet is an annual tropical grass found to be very resistant to low soil moisture situations. It is thought to have originated in tropical Africa and is cultivated as a food grain in Africa and India. It is used in the Southern coastal plain of the United States for temporary summer grazing (35). The scientific name of pearl millet has changed over the past two decades and the literature bears such names as Pennisetum glaucum, P. typhoideum, P. typhoides (Burm) and presently P. americanum (L.) K. Shum (15,35).

At maturity, plants of pearl millet range from 1.5 to 3 meters in height and have flat leaf blades which reach to 1 meter in length.

Panicles of this grass are stiff and cylindrical from 20 to 45 cm in length and are similar in size and shape to common cattail growing in marsh areas. Panicles possess short-pedicled, two-flowered spikelets with the lower floret imperfect and usually staminate. The upper floret is fertile and perfect with the caryopsis surrounded by a bristled involucre (35).

Pearl millet is highly cross-pollinated with its stigmas being exerted several days before the anthers. This flowering characteristic facilitates making hybrids in a plant breeding program. Heterosis has resulted in increased forage yields of up to 71% when hybrids are compared to their inbred parents. But even with its cross-pollinated character, selfed seed is easily obtained by bagging (15, 35).

Pearl millet is generally a diploid with a basic chromosome number of 7. Its chromosome pairing at metaphase I was studied and found to give 59.3% closed bivalents, 34% open bivalents and 6.8% univalents. Autotetraploids were induced and found to be less fertile than diploids. Autotriploids are produced by crossing tetraploids to diploids. Autotriploids will occur spontaneously from fertilization of unreduced female gametes (15).

Interspecific hybrids can be produced with pearl millet. It is generally easier to obtain these hybrids with those species having the same basic chromosome number. Pennisetum purpureum (4x=28) has been hybridized successfully with pearl millet (15).

Burton (12) found plants from pearl millet crosses involving inbred 556 x inbred 23 failed to shed pollen or set selfed seed. Seed were set if dusted with pollen from inbred 23. Plants from the backcross, (556 X 23) X 23, shed no pollen or selfed seed yet when pollinated with other lines readily set seed. This was the first recorded case of cytoplasmic male sterility (CMS) in pearl millet. Further work resulted in the release of the male sterile inbred lines 23A and 23DA and their maintainers 23B and 23DB (14). Several other sources of CMS lines have also been located (16). This characteristic has made possible commercial production of hybrid pearl millet seed.

Spontaneous mutations from male sterile to male fertile plants were observed and thought be be both cytoplasmic and genetic in nature (13). Subsequent studies on four different male sterile sources indicated the mutations to be cytoplasmic with mutation rates of 0.15 to 1.02/100 seedheads greatly influenced by the plant's genotype (16).

MATERIALS AND METHODS

Fifteen inbreds and five hybrids of pearl millet were obtained (courtesy Dr. Glenn Burton, USDA-ARS, Tifton, GA) for use in screening for inoculation response. The sixth hybrid (23 DA x PMB004, courtesy Jimmy Barber, North American Plant Breeders, Hutchinson, Kansas) was the female of the three-way cross shown to respond to Spirillum inoculation (45). All genotypes are shown in Table 1. The A lines are cytoplasmic male sterile and B lines are maintainers for them. D lines carry the dwarf gene. Any commercial hybrid is listed in parenthesis by its varietal name.

Inoculum was Spirillum lipoferum, strain Sp 13t (courtesy Dr. Johanna Dobereiner, Empresa Brazileira de Pesquisa Agropecuaria, Rio de Janeiro, Brazil) grown in sealed 12 l glass containers with aeration in either nitrogen free or nitrogen containing liquid medium (Table 2). To standardize the Sp 13t inoculum applied in different experiments, cells were first cultured in rich peptone medium for 24 hours, centrifuged and supernatant removed. Peptone medium was 15% glycerol substituted for the water was added to an equal volume of cells and sedimented cells resuspended. This heavy suspension (10^{11} cells) was placed in 1 ml aliquots in Cooke pro-vials and stored under liquid nitrogen (courtesy Dr. Max E. Tyler, Department of Microbiology, University of Florida, Gainesville, FL).

All fertilizer was applied at a calibrated per hectare rate of 60 kg N (as NH_4NO_3), 7.9 kg P, 30.1 kg K and 5.5 kg of fritted trace

TABLE 1

PEARL MILLET INBREDS AND HYBRIDS USED IN SCREENING FOR RESPONSE TO Sp 13t INOCULUM IN GREENHOUSE AND FIELD STUDIES

INBREDS

Tift 23DA	Tift 186	Tift 131
Tift 23DB	Tift 239B	Tift 123
Tift 23A	Tift 383	Tift 18DB
Tift 23B	Tiftlate	Tift 13
Tift 239A	Tift 23SB	Bil 3B

HYBRIDS

Tift 23DA x Tiflate
Tift 23DA x Tift 18DB
Tift 23DA x Tift 186 (Gahi 3)
Tift 23DA x Tift 383 (Tifleaf)
Tift 23DA x PMB004
Tift 23A x Bil 3B

TABLE 2

COMPOSITION OF NITROGEN FREE AND NITROGEN CONTAINING MEDIA USED TO PRODUCE ALL LIQUID BACTERIAL INOCULUM

	N Free* g/liter	N containing g/liter
D-L Malic Acid	5	5
NaCl	0.1	0.1
$MgSO_4 \cdot 7\ H_2O$	0.2	0.2
$CaCl_2$	0.02	0.02
$FeCl_3$	0.01	0.01
K_2HPO_4	0.1	6
KH_2PO_4	0.4	4
$NaMoO_4 \cdot 2H_2O$	0.002	0.002
Yeast extract	----	0.05
NH_4Cl	----	1
Tryptocase(16% N)	----	5

*From Dobereiner and Day (21)

elements (5 g B, 5 g Cu, 29 g Fe, 12 g Mn, 0.3 g MO, and 11 g Zn). Fertilizer rates and Sp 13t were selected on the basis of previous success of enhancing growth in pearl millet with these two parameters as variables (45).

Soil used in greenhouse and field studies was an Arredondo loamy fine sane, pH 6.0 to 6.5. Any soil autoclaving was achieved at .844 kg/cm^2 steam pressure for five hours then repeated 24 hours later.

Standardized Procedures

Throughout this investigation, increases in plant dry weight, percent nitrogen, total nitrogen, and acetylene reduction were used as predictors of N_2-fixation achieved through Sp 13t inoculation. Data were statistically analyzed by analysis of variance and comparisons between means made by LSD. Mention of a trademark or proprietary product does not constitute or guarantee warranty of the product, and does not imply its approval to the exclusion of other products that may be suitable.

Dry weight measurements were determined on soil and plant tissue dried at 60 C for 48 hours in a forced air drier. Dried material was then prepared for nitrogen analyses. Soil was screened four times through a 2 mm screen and plant tissue was ground in a 1 mm mesh Wylie mill and/or a 1 mm mesh disintegrator (Christy Norris LTD, Chelmsford, England). Material was stored in sealed, plastic bags in a freezer (-12 C) until ready for use.

All nitrogen determinations were conducted by first performing a block digestion (26) on 2 g of the screened soil or .1 g of the ground

plant tissue. This digestion was conducted in 10 ml of concentrated H_2SO_4, 2 ml of concentrated H_2O_2, 3.5 gm of catalyst-salt mixture (90% K_2SO_4 : 10% $CuSO_4$), and 2 to 3 boiling chips for 1.5 hours on an aluminum block heated to 400 C (25). The digestate was then diluted to 75 ml with distilled water and analyzed by a Technicon nitrogen autoanalyzer for nitrogen concentration.

Acetylene reduction assays were conducted on root-soil cores or preincubated, excised washed roots. Preincubation denotes that time before acetylene is introduced into the chamber containing the roots. Underestimations of acetylene reduction in freshly washed roots are reported due to a lag period in nitrogenese activity. Preincubation of washed roots is conducted to overcome this problem (27). But preincubation is believed to overestimate predictions of quantities of nitrogen fixed possibly due to increases in microbial populations (1). The core method was used to overcome the preincubation problems and was thought to offer least disturbances of root-bacteria systems.

Washed roots were sealed in plastic bags, then preincubated overnight in an atmosphere of 5% O_2 - 95% N_2 at 30 C. Plastic bags contained a rubber septum taped on its side to allow exchanging of gases. After preincubation, the atmosphere was replaced with a fresh O_2 - N_2 mixture and 10% acetylene added. After three hours incubation at 30 C, samples (11 cc) were taken, placed in 8 cc evacuated tubes, and subsequently analyzed by gas chromatography. Details of chromatographic analysis have previously been reported (27).

Core samples were prepared as follows: The top of pearl millet plants were cut away at ground level. A soil core containing the roots were removed by inserting a metal collar (made by removing both ends of a soft drink can and sharpening the bottom rim) over the plant stubble, forcing the collar into the soil, and then removing the soil core along with the metal collar. The intact soil core with metal collars was placed in glass jars with a void volume of 473 ml. Glass jars were sealed with metal lids and threaded rims (Mason jars). Metal lids contained a sealed rubber septum for gas exchange. The atmosphere was replaced with gas containing partial pressures of 0.86 atm N_2, 0.05 atm O_2 and 0.09 atm acetylene. After a four-hour incubation at 30 C, samples were taken and analyzed as described in the washed root procedure.

Screening Trials

Greenhouse screenings were conducted to supplement field screening trials. It was believed that the field trials represented the best way to screen for inoculation response since a plant achieves maximum growth and development in the field. But field experiments require much time and space to conduct, whereas planting in small greenhouse containers allows screening of many plants anytime during the year. Also, the greenhouse screening studies permit testing the effects of added organic matter and nitrogen to the system. Genotype responses in the greenhouse flat and pot studies were compared to their field results.

Greenhouse Screening

Two types of greenhouse screening experiments were conducted: one in flats, the other in pots. In the experiments conducted in flats, Tift inbreds 123, 13, 23 SB and 131 were used. Seed of each inbred were planted in rows at a rate of .5 g/m of row in the 54 cm x 38 cm x 6 cm flats. Each flat contained either autoclaved field soil analyzed at 1.3% organic matter and 0.05% nitrogen or an autoclaved field soil amended with manure and decomposed organic matter to analyze at 3.5% organic matter and 0.10% nitrogen. All flats were given standard fertilizer rates. Seven days after planting, 30 ml of Sp 13t inoculum, grown under standard conditions in nitrogen free medium to approximately 10^7 cells/ml, were drenched onto each row within each flat. Control flats received the same rate of autoclaved inoculum in the same manner. Treatments were thus a factorial combination, inbred X soil type X inoculum, at one flat each. Plant tops were harvested for dry matter 28 days after inoculation. A washed root acetylene reduction assay was also conducted at harvest on plants grown under each treatment. The experiment was repeated one month later but with two flats per treatment.

In the pot experiments, four half-sib hybrids, 23DA X PMB004, 23DA X 383, 23DA X 186, and 23DA X 18DB, and the maintainer line 23DB were germinated in cell packs. Four uniform seedlings were then transplanted into 25 cm plastic pots containing autoclaved field soil from the nursery area where the field screenings were conducted. Pots containing the plants were given standardized fertilizer rates. Three

days later, 25 ml treatments of Sp 13t inoculum or autoclaved inoculum, grown under standard conditions in nitrogen free media to approximately 10^7 cells/ml, were injected by a needle and syringe into the root zone of respective plants. Treatments were thus a factorial combination, genotype X inoculum at three replications each. After a 21-day regrowth, plants were harvested a second time for dry matter.

Field Screening

All inbreds and hybrids were tested in a field production nursery for enhanced plant dry matter, nitrogen, and acetylene reduction to Sp 13t inoculation. The experiment was conducted in split-plot design with genotypes as a six row main plot, replicated three times. All rows were 2.7 meters long. Subplots of Sp 13t inoculum or autoclaved inoculum were drenched onto the second or fifth row with watering cans then lightly irrigated to wash the bacteria into the soil. Inoculum was grown under standard conditions in nitrogen containing medium and applied at the rate of 4 X 10^7 cells/cm of row. Inoculum treatments were reapplied two weeks later with a small push plow rigged with a CO_2 constant delivery sprayer. This device injected the liquid into the plant's root zone through a nozzle behind the furrow opener. Injection was used to avoid the need to irrigate as with the drench method. Also, this sprayer delivered a constant supply of liquid over a given area at a pressure of 3.57 kg/cm^2.

Seed of each genotype were planted at a rate of .5 g/m of row with a belt planter. Standard fertilizer and inoculum treatments

were applied after seedling emergence. General maintenance of hand weed control, irrigation, and insect pest control with Lanate were used as needed. At anthesis (70 to 80 days) plants were sampled for acetylene reduction by both core and excised washed root assays. Sampling at full anthesis was reported to be the best time to sample for highest activity in other grass - Spirillum systems (47). Four random plants per treatment row were taken for percent nitrogen determinations. The complete row was harvested with a Carter flail harvester and total wet forage weighed. A subsample of the forage was taken for dry weight calculations.

Nitrogen Balance Study

Hybrid 23DA X 186, that responded to Sp 13t inoculation with increased yield in the field screening trials was subjected to a nitrogen balance study. Inputs of nitrogen due to inoculations into the plant - soil system were monitored.

Glazed, ceramic containers, 23 cm in diameter and 30.5 cm deep, with a drain in the bottom for catching leachable liquid were used in this study. Nonautoclaved bulk-mixed soil from the field nursery area was weighed for each container and a sample taken with a 2.5 cm probe for soil dry weight determination. Standard fertilizer rates were added to the soil and mixed in a clean cement mixer for 15 minutes per container. Two uniform seedlings of 23DA X 186 were transferred into each container and 100 ml of inoculum or autoclaved inoculum were injected into each container with needle and syringe. Inoculum was grown under standard conditions to 10^8 cells/ml in nitrogen containing

medium. After 24 hours, five soil samples were taken from each container with a 2.5 cm probe through the soil profile to give the initial nitrogen determinations for each container. The experiment thus consisted of two treatments, inoculum and autoclaved inoculum, as pairs within each of 18 blocks. Throughout the study, each pair was rotated weekly within its block to overcome border effects. Light was supplemented from incandescent and flourescent sources at the end of the day to achieve a 13.5 hour photoperiod. Plants were hand watered once a day to completely wet soil profile. Any leachable liquid was collected and returned to its respective container.

After 42 days growth, five soil samples were taken as before. Plants were then removed from their containers and washed free of remaining soil. A small sample of roots was excised for a washed root acetylene reduction assay. This washed root assay differed from the one previously described in that no preincubation was used, argon was substituted for the 95% N_2 - 5% O_2 air mixture and samples were taken directly for chromatography at 17 and 24 hours. Soil and plant tissue was dried and prepared for nitrogen analysis.

RESULTS AND DISCUSSION

Screening Trials

Greenhouse Screening

Results of the experiment conducted in flats are presented in Table 3. Data from the two studies which were repeated over time were composited as replications. This was done to give a truer representation of inoculation response as the number of replications at each time were limited. Trends of increasing dry weight are found within each soil type. Acetylene reduction activity was similar within amended field soil but decreased in the nonamended field soil.

Data for the experiment conducted in pots are presented in Table 4. In harvest 1, inoculum significantly (LSD = 0.35, p = 0.05) enhanced overall dry weight (data pooled across all hybrids). This trend was significantly reversed (LSD = 0.56, p = 0.05) in favor of autoclaved inoculum on regrowth to havest 2.

Limited replications and possible contamination of control treatments probably contributed to the erratic greenhouse screening data. These problems resulted in measuring no differences among inbreds and hybrids for response to Sp 13t inoculum. All statistically significant inoculum responses occurred in overall means for each treatment as these data have more replications (pooled across all genotypes).

TABLE 3

DRY WEIGHT AND ACETYLENE REDUCTION RESPONSE OF PEARL MILLET INBREDS TO INOCULATION WITH Sp 13t. CONTROL TREATMENTS WERE AUTOCLAVED Sp 13t. PLANTS WERE GROWN IN FLATS IN THE GREENHOUSE AND EFFECTS OF TWO AUTOCLAVED SOIL TYPES (FIELD SOIL ANALYZED AT 1.3% ORGANIC MATTER AND 0.05% NITROGEN AND FIELD SOIL AMENDED WITH MANURE AND DECOMPOSED PEAT TO ANALYZE AT 3.5% ORGANIC MATTER AND 0.10% NITROGEN) WERE ALSO INVESTIGATED.

	Field Soil				Amended Field Soil			
	Dry Weight (g/plant)		Acetylene Reduction (nmole/g dry root x hr)		Dry Weight (g/plant)		Acetylene Reduction (nmole/g dry root x hr)	
Inbred	Inoc	Auto Inoc	Inoc	Auto Inoc	Inoc	Auto Inoc	Inoc	Auto Inoc
Tift 123	.65	.33	46	156	1.57	1.04	38	58
Tift 13	1.33	1.17	49	205	2.00	1.86	32	12
Tift 23SB	1.04	.88	91	129	1.80	2.00	30	5
Tift 239B	.74	.60	88	261	1.61	1.81	121	0
Tift 131	1.52	.71	92	124	1.70	1.22	26	27
Overall $\bar{X}$	1.05	.74	73	175	1.75	1.58	49	20

TABLE 4

DRY WEIGHT RESPONSE OF PEARL MILLET HYBRIDS TO INOCULATION WITH Sp 13t. CONTROL TREATMENTS WERE AUTOCLAVED Sp 13t. PLANTS WERE GROWN IN THE GREENHOUSE IN POTS CONTAINING AUTOCLAVED FIELD SOIL

Hybrid	Harvest 1 (g/plant)			Harvest 2 (g/plant)			Cumulative Total (g/plant)		
	Inoc	Auto Inoc	Difference	Inoc	Auto Inoc	Difference	Inoc	Auto Inoc	Difference
23DA X PMB004	3.1	2.3	+0.8	3.6	4.8	-1.2	6.7	7.1	-0.4
23DA X 383	4.0	3.5	+0.5	1.7	1.8	-0.1	5.7	5.3	+0.3
23DA X 186	3.2	3.2	0	2.0	3.2	-1.2	5.2	6.4	-1.2
23DA X 18DB	3.4	2.9	+0.5	3.1	3.5	-0.4	6.5	6.4	+0.1
23DB	2.2	2.0	+0.2	1.7	2.5	-0.8	3.9	4.5	-0.6
Overall $\bar{X}$	3.2	2.8	+0.4*	2.4	3.2	-0.8**	5.6	5.9	-0.3

* LSD = 0.35, p = 0.05.

** LSD = 0.56, p = 0.05.

Field Studies

Agronomic dry weight, percent nitrogen, and total nitrogen for the hybrids are presented in Table 5. The 23DA X 186 ('Gahi 3') hybrid gave a significant response in dry weight (LSD = 2372, $p = 0.05$) to Sp 13t inoculation when compared to its autoclaved control. In this hybrid, the autoclaved control yielded approximately 13 MT/ha over 80 days with inoculation enhancing dry weight to approximately 17 MT/ha for a 32% increase. A 37% increase in total plant nitrogen was brought about by inoculation in this hybrid. Other hybrids did not respond significantly to inoculation for any agronomic parameter. Inbred dry weight, percent nitrogen, and total nitrogen data are shown in Table 6. It is noted that enhanced responses in dry weight of 1192 and 1151 kg/ha are seen in inbreds Bil 3B and Tiflate respectively. This would indicate trends that warrant further testing.

The hybrid, 23DA X 186 ('Gahi 3'), responded in dry weight to inoculation while its parents showed no response. If the degree of heterosis of hybrids over their highest yielding parent is examined in Table 7, 'Gahi 3' exhibits a low degree of heterosis compared to other hybrids in the study. It appears from these results that the degree of hybrid vigor or heterosis would not be a good predictor of plant response to inoculation, but rather the specific plant genetic might be. This is especially interesting when compared to work in wheat by Neal and Larson (37) where an asymbiotic bacillus possessing nitrogenase activity was consistently isolated from specific substitution lines while no bacilli were found in either of the two parents.

TABLE 5

AGRONOMIC YIELD IN DRY MATTER, PERCENT NITROGEN, AND TOTAL NITROGEN OF PEARL MILLET HYBRIDS FROM THE FIELD SCREENING TRIALS

Hybrid	DRY MATTER - Kg/ha			% NITROGEN			TOTAL NITROGEN - Kg/ha		
	Inoc(+)	Auto Inoc(-)	Diff.	Inoc(+)	Auto Inoc(-)	Diff.	Inoc(+)	Auto Inoc(-)	Diff.
Tift 23DA x Tift 18DB	11234	10900	+ 335	1.73	1.74	- .01	194	191	+ 3
Tift 23DA x Tiflate	17858	19588	-1730	1.58	1.43	+ .15	282	280	+ 2
Tift 23DA x Tift 383	10712	11523	- 812	2.02	2.01	+ .01	215	231	- 16
Tift 23DA x Tift 186	17269	13116	+4153*	1.57	1.50	+ .07	272	198	+ 74
Tift 23DA x PMB 004	11241	11791	- 551	1.62	1.65	+ .03	180	195	- 15
Tift 23 A x Bil 3 B	9229	8978	+ 251	1.60	1.68	- .08	151	151	0

*LSD = 2372, p = 0.05

TABLE 6

AGRONOMIC YIELD IN DRY WEIGHT, PERCENT NITROGEN, AND TOTAL NITROGEN OF PEARL MILLET INBREDS FROM THE FIELD SCREENING TRIALS

Genotype	Dry Matter Kg/ha			% Nitrogen Kg/ha			Total Nitrogen Kg/ha		
	Inoc.	Auto Inoc.	Diff.	Inoc.	Auto Inoc.	Diff.	Inoc.	Auto Inoc.	Diff.
Tift 23DA	7529	7647	- 118	2.10	1.89	+ .21	158	145	+ 13
Tift 23DB	6168	7018	- 850	2.01	1.94	+ .07	124	136	- 12
Tift 23A	7927	8269	- 342	1.78	1.71	+ .07	141	141	0
Tift 23D	8545	8961	- 416	1.65	1.59	+ .16	140	141	- 1
Tift 239A	6777	6726	+ 48	1.90	1.91	- .01	129	128	+ 1
Tift 239B	7354	6519	+ 835	1.83	1.75	+ .08	134	115	+ 19
Tift 186	10368	11363	- 995	1.91	1.97	- .06	198	224	- 26
Tift 383	8030	7400	+ 638	1.96	2.05	- .09	155	153	+ 2
Tiflate	16012	14861	+1151	1.55	1.48	+ .07	248	220	+ 28
Tift 23SB	9527	10113	- 586	1.63	1.65	- .02	155	168	- 13
Bil 3B	8023	6831	+1192	1.47	1.53	- .06	118	105	+ 13
Tift 13	7859	8738	- 879	1.52	1.54	- .02	117	134	- 17
Tift 131	9271	8749	+ 522	1.59	1.80	- .21	148	155	- 7
Tift 123	10050	9856	+ 194	1.89	1.91	- .02	187	187	0
Tift 18DB	6001	6457	- 456	2.24	2.07	+ .17	134	133	+ 1
$\bar{X}$	8629	8634	- 5	1.80	1.79	+ .01	152	152	0

TABLE 7

PERCENT HETEROSIS OF HYBRIDS OVER THEIR HIGHEST YIELDING PARENT AS CALCULATED FROM THE CONTROL (AUTOCLAVED INOCULUM) TREATMENT

Hybrid	% Heterosis
23DA X 18DB	42.5
23DA X Tiflate	31.8
23DA X 383	50.7
23DA x 186	15.4
23DA X Bil 3B	8.6

They suggested that the genetics of the wheat plant might play a large role in altering establishment of such bacilli in its root environment.

Acetylene reduction values in both inbreds and hybrids were low (range 0-54 nmole/g dry root x hr) and did not support the yield parameters (Table 8). Plots were not sampled extensively for acetylene reduction because of the damage it would cause agronomic yields. Acetylene reduction only measures nitrogenase activity during the time of the assay and the active time of the enzyme might have been missed. The possibility therefore exists that N_2-fixation was not measured due to the limited samplings (once for each assay at harvest).

Nitrogen content (percent nitrogen) was not increased by inoculation. Normally one expects nitrogen content to increase with better nitrogen nutrition of the plant, but percent nitrogen has been reported to drop in some tropical forage grasses when nitrogen fertilization was increased to 200 kg N/ha (46). Total nitrogen per unit of area might therefore be a better predictor of added nitrogen to a grass especially in the range of nitrogen inputs of any associative N_2-fixing system. But total nitrogen cannot be the only predictor of N_2-fixation because it will increase proportional to dry weight. Other mechanisms besides N_2-fixation will cause a plant to accumulate dry matter (7).

The experimental design of confining inoculum and autoclaved inoculum treatments to paired subplots was thought to reduce plot to plot variation and allow use of less replication for better land utilization. A significant interaction was then defined as differences

TABLE 8

ACETYLENE REDUCTION VALUES BY CORE AND WASHED ROOT ASSAYS OF INBREDS AND HYBRIDS FROM THE FIELD SCREENING TRIALS

	Core Acetylene Redcution (nmole/g dry root x hr.)			Washed Root Acetylene Reduction (nmole/g dry root x hr.)		
Inbreds	Inoc.	Auto Inoc.	Diff.	Inoc.	Auto Inoc.	Diff.
Tift 23DA	2.0	3.0	- 1.0	13.0	9.0	+ 4.0
Tift 23DB	6.3	1.0	+ 5.3	0	10.5	-10.5
Tift 23A	7.0	4.6	+ 2.4	3.5	0	+ 3.5
Tift 23D	8.0	1.3	+ 6.7	27.5	14.5	+13.0
Tift 239A	0	0	0	9.0	10.5	- 1.5
Tift 239B	0	0	0	11.0	6.0	+ 5.0
Tift 186	0.3	1.6	- 1.3	10.0	11.0	- 1.0
Tift 383	1.6	6.3	- 4.7	4.5	1.0	+ 3.5
Tiflate	0	0	0	15.5	7.0	+ 8.5
Tift 23SB	2.0	4.3	- 1.3	12.5	3.0	+ 9.5
Bil 3B	0	0	0	6.0	0	6.0
Tift 13	0	0	0	8.5	11.5	- 3.0
Tift 131	7.6	13.0	- 5.4	10.5	7.5	+ 3.0
Tift 123	6.7	2.6	+ 4.1	11.5	3.5	+ 8.0
Tift 18DB	0	0	0	9.5	10.0	- 0.5
Hybrids						
23DA X 18DB	2.0	6.0	- 4.0	4.5	1.5	+ 3.0
23DA X Tiflate	0	0	0	5.5	2.0	+ 3.5
23DA X 383	27.0	25.3	+ 1.7	14.0	7.5	+ 6.5
23DA X 186	1.0	2.0	- 1.0	0	0	0
23DA X PMB004	23.7	8.3	+15.4	31.5	12.0	+19.5
23A X Bil 3B	54.3	15.0	+19.3	14.0	8.0	+ 6.0

among genotypes to respond to inoculation. If the effect in any one genotype is small but consistent, it could be masked by the random variation created by many genotypes not responding in the population. Another method of locating one or two responding genotypes would be the use of outlier tests such as the Grubbs Test (28). Outlier tests allow screening for those outling values which are not predicted by the interaction term. In the inbred population, it was found that a difference in dry weight of 1736 kg/ha was needed for any genotype to be an outlier ($p = 0.05$). Even with this test, no response is observed with Bil 3B (1192 kg/ha) or Tiflate (1151 kg/ha). But 1736 kg/ha represents a 25% increase in Bil 3B and only a 12% increase in Tiflate. This is a discrepancy in this experiment to measure individual responding genotypes. Screening within a population with wide yield variability might not be a valid approach when inoculation response emphasizes enhancement of the same yield parameters.

Results of the field and greenhouse studies indicate that a schedule to screen for associative N_2-fixation should place initial emphasis on experiments designed to show increased nitrogenase activity achieved through inoculation. Well replicated genotypes could be screened each cycle of selection by extensive sampling with acetylene reduction or if possible, $^{15}N_2$. Responders should then be field tested individually for increases in agronomic yield and acetylene reduction.

Nitrogen Balance Study

Though the 32% yield increase by inoculated 'Gahi 3' is important agronomically, data did not show adequate N_2-fixation to explain this

increase. There are also reports that Sp 13t is in a group of Spirillum lipoferum strains which show potential for dinitrification (38). This nitrogen balance study was conducted to monitor increases of nitrogen in inoculated 'Gahi 3' plants and to verify its field growth responses.

The effects of Sp 13t inoculum on 'Gahi 3' plants are seen in Table 9. Increases from .5 to 4.7% are found in the various plant parameters due to inoculation. Only total plant dry weight ($p = 0.28$) and total plant nitrogen ($p = 0.21$) showed any noticeable statistical response. This experiment was conducted for a 42 day growth period, while in the field screening trials plants were grown for 80 days. At termination of this nitrogen balance study plants were yellow and seemed to be uniformly deficient in nitrogen, but possibly a longer growth period was needed to duplicate the statistically significant ($p = 0.05$) field yield enhancements.

The nitrogen balance for the soil-plant system is presented in Table 10. Inoculation with Sp 13t did not increase the nitrogen content for the total system. It is interesting that an overall experimental nitrogen increase of 0.2628 g (combining all treatments) is seen. This amount of nitrogen represents 3.48% of the initial soil nitrogen and might be viewed as the sampling error within this nitrogen balance study. From previous work with pearl millet, it was predicted that Sp 13t inoculum could replace 39 kg N/ha if 60 kg N/ha was added as fertilizer and plants achieved a 22% increase in yield (45). This soil was found to have about 0.055% nitrogen; and

TABLE 9

DRY WEIGHT AND NITROGEN RESPONSE OF 23DA X 186 PLANTS TO INOCULATION WITH Sp 13t IN THE NITROGEN BALANCE EXPERIMENT

Treatment	dry weight (g)			% N		g N		
	Top	Root	Total	Top	Root	Top	Root	Total
Live Sp 13t	39.67	16.89	56.55	0.702	0.717	0.2784	0.1193	0.3977
Autoclaved Sp 13t	38.67	16.11	54.78	0.698	0.704	0.2675	0.1139	0.3814
% Increase Due to Inoc.	2.5	4.9	3.2	0.5	1.8	4.1	4.7	4.3

TABLE 10

NITROGEN BALANCE OF THE 23DA X 186 - SOIL SYSTEM
IN THE NITROGEN BALANCE EXPERIMENT

Treatment	Soil N (g)			Plant N (g)	Balance (g N) (Plant N - Soil N Lost
	Initial	Terminal	Lost (Initial-Terminal)		
Live Sp 13t	7.5693	7.4047	0.1645	0.3977	+0.2332
Autoclaved Sp 13t	7.5228	7.4339	0.0889	0.3814	+0.2925
$\bar{X}$ For Combined Treatments	7.5460	7.4194	0.1267	0.3895	+0.2628

if one assumes that an acre furrow slice of soil weighs 2,000,000 lbs. (9), then there would be 1171 kg of nitrogen after fertilizer addition. Therefore the predicted increase of 39 kg N/ha would be about 3.33% of this total. It does appear that the amount of nitrogen that could be fixed was within the sampling error of the experiment. This supports the predictions of total nitrogen determinations not being sensitive enough to show small levels of N_2-fixation (11). Also, the low levels of plant yield possibly negated finding a nitrogen increase for the system. As there was no overall decrease in nitrogen, dinitrification resulting in loss of nitrogen from the system was not measured. More sensitive assay such as ^{15}N should be used to prove whether N_2-fixation is taking place in the 'Gahi 3' - Sp 13t system.

Plant root acetylene reduction values are presented in Table 11. Inoculation with live Sp 13t cells enhanced acetylene reduction by 21 nmoles/g dry root x hr and are of some statistical value when compared to autoclaved controls ($p = 0.21$). This level of increased nitrogenase activity attained with the hybrid is encouraging, but, as stated previously, limited sampling does not allow predicting N_2-fixation for the whole term of the experiment.

TABLE 11

ACETYLENE REDUCTION VALUES ON TREATMENTS IN THE NITROGEN BALANCE STUDY. SAMPLES WERE TAKEN AT TERMINATION OF THE EXPERIMENT, INCUBATED IN ARGON WITH 10% ACETYLENE, AND SAMPLES ANALYZED BY GAS CHROMATOGRAPHY AT 17 AND 24 HOURS

Treatment	17 Hours (nmole/g dry root x hr)	24 Hours (nmole/g dry root x hr)
Live Sp 13t	84.89	108.94
Autoclaved Sp 13t	72.78	87.11

CONCLUSIONS

Differences among pearl millet inbreds and hybrids to respond to Sp 13t liquid inoculation were found. A hybrid, 23DA X 186 ('Gahi 3'), responded in the field with a 32% increase in dry weight and a 37% increase in total nitrogen while its parents showed no response. Heterosis is not thought to be the sole cause of this response. An inbred, Bil 3B, was enhanced 17% in dry weight by Sp 13t inoculation and has potential for further study.

All screening studies reported in this investigation placed emphasis on increased plant dry weight, percent nitrogen, total nitrogen, and acetylene reduction achieved through inoculation. Of these acetylene reduction represents the most sensitive assay for indicating increased nitrogenase activity. In a population with wide yield variation it is difficult to predict N_2-fixation on any one genotype when response is measured by further yield enhancements. Screening pearl millet for associative N_2-fixation achieved through inoculation should place initial emphasis on well replicated experiments designed to show increased nitrogenase activity by extensive sampling with acetylene reduction or, if possible, $^{15}N_2$. Those genotypes which are deemed responders should then be field tested individually for increases in agronomic yield and acetylene reduction.

Liquid inoculation of 'Gahi 3' did result in very substantial increases in dry weight and nitrogen. A nitrogen balance study with

this hybrid did not indicate that the growth enhancements were based on significant amounts of nitrogen added into the plant-soil system. It is possible that any N_2-fixation achieved through inoculation might have been within the limits of sampling error for the nitrogen balance system. Sensitive assays such as $^{15}N_2$ are needed to confirm reconstitution of N_2-fixation by Sp 13t inoculation in the 'Gahi 3' hybrid.

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He is married to the former Mary Jeanne Carr and they have a daughter, Melinda.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rex L. Smith, Chairman
Associate Professor of Agronomy

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